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Short communications

The single epsin homolog in *Giardia lamblia* localizes to the ventral disk of trophozoites and is not associated with clathrin membrane coats

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ABSTRACT

Epsins serve as recruitment platforms for clathrin membrane coat protein components and induce membrane curvature via their N-terminal homology (ENTH) domain. Unexpectedly, the single ENTH domain protein, a putative epsinR homolog (Glepsin), in the diverged protozoan parasite *Giardia lamblia*, localizes exclusively to the specialized attachment organelle, the ventral disk (VD). Glepsin binds both to phosphatidylinositol (3,4,5)-trisphosphate phospholipids and the VD cytoskeleton, but lacks canonical domains for interaction with clathrin coat components. This suggests reassignment of giardial epsin function from membrane trafficking to a structural role in linking the plasma membrane to the highly specialized VD during evolution of this genus.

*Keywords***:** Epsin, *Giardia*, attachment, ENTH-domain, lipid binding, ventral disk

To prepare for survival in the environment, the protozoan parasite *Giardia lamblia* transforms from a flagellated form into a cyst before being shed by its mammalian host. Encystation entails synthesis and regulated secretion of a composite biopolymer matrix composed of three cyst wall proteins (CWPs) and glycan (Samuelson *et al.*, 2013). Cyst wall material (CWM) is selectively accumulated and matured in encystation-specific vesicles (ESVs) (Konrad *et al.*, 2010). Even though ESVs along with all other giardial organelles appear to be fixed in position in trophozoites (Gaechter *et al.*, 2008; Konrad *et al.*, 2010; Stefanic *et al.*, 2009), free exchange of fluid phase material containing cyst wall proteins between organelles is ensured by highly dynamic tubular membrane structures (Stefanic *et al.*, 2009). In *Giardia*, two genes code for factors that are potentially able to induce formation of membrane tubules and recruit effectors to the cytoplasmic side of membranes (Hinshaw and Schmid, 1995; Horvath et al., 2007; Sweitzer and Hinshaw, 1998). A dynamin-like protein (Gaechter *et al.*, 2008) and an ENTH (epsin N-terminal homology) domain protein (Kay *et al.*, 1999) (*Giardia* genome database accession number GL50803_3256). The single giardial dynamin may play a general role in organelle morphogenesis, since conditional expression of a dominant-negative variant led to massive enlargement of both ESVs and *Giardia*-specific endosomal-lysosomal compartments termed peripheral vesicles (PVs) (Gaechter *et al.*, 2008). However, formation of membrane tubules allowing inter-organelle protein exchange does not seem to depend on dynamin function (Hehl, Stefanic & Gächter, unpublished). Here, we set out to test whether the only ENTH domain containing protein, which is predicted to bind to lipid membranes and induce membrane curvature, localized to ESVs and could play a role in formation of membrane tubular inter-organelle connections.

Mammalian epsin proteins contain a highly conserved N-terminal ENTH domain and function as adaptors providing interaction platforms for various components of clathrin-dependent endocytosis such as recognition and selection of cargo (Horvath *et al.*, 2007). Four epsin isoforms have been characterized in mammals. Their ENTH domains

are characterized by seven alpha helices, one of which, the alpha-0 helix, can insert into the cytoplasmic leaflet of the lipid bilayer and is capable of inducing membrane curvature (Ford *et al.*, 2002). The three canonical epsins 1,2,3, named after their ability to interact with epidermal growth factor receptor substrate 15 (Eps15) via their C-terminal domains, bind to adapter protein complex (AP) 2 as well as clathrin and are involved in clathrin-mediated endocytosis (Mills *et al.*, 2003). Furthermore, they can bind to ubiquitinated proteins via a conserved ubiquitin interacting domain (UID) (Fig. 1A). A fourth non-canonical epsin, epsinR, lacks an UID and does not bind to Eps15 (Mills *et al.*, 2003). Instead, epsinR proteins contain an AP/GGA (Golgi-localized, gamma-ear–containing ADP-ribosylation factor–binding protein) binding motif with a role in AP1 recruitment (Mills *et al.*, 2003). Mills *et al.* suggested that epsinR is involved in clathrin-mediated budding of vesicles destined for endosomes from the trans-Golgi network (Mills *et al.*, 2003). Hence, both the canonical epsins and epsinR have a similar, albeit compartment-specific, role as adaptors in clathrin-mediated transport. EpsinR homologues harbor a distinctive C-terminal methionine-rich domain with an unclear function (Horvath *et al.*, 2007). The open reading frame GL50803_3256 encodes a predicted protein of 405 amino acids with conserved domains and significant similarity to epsin family proteins, henceforth called *Giardia lamblia* epsin (Glepsin). Glepsin has a conserved N-terminal ENTH domain (e-value = 8.50e-25) and a C-terminal methionine and proline-rich region. Compared with other epsinR homologs, there is also a strikingly high proportion of glutamine stretches in the C-terminal half of Glepsin.

To test whether Glepsin was associated with organelle membranes or even membrane tubules in encysting trophozoites, a C-terminally hemagglutinin (HA)-tagged full-length Glepsin variant (construct GlepsinFl-HA) was conditionally expressed in transgenic *G. lamblia* under the control of the cyst wall protein 1 (CWP1) promoter (Fig. 1). The GlepsinFl-HA product was predominantly detected in the cytoplasm of induced trophozoites with some association to cytoskeletal structures (Fig. 1Ba) but not to ESV, PV organelles or the endoplasmic reticulum (ER). In many cases, GlepsinFl-HA appeared to form large aggregates, i.e. one or several round <1µm structures per cell (Fig. 1Ba). To investigate if the ENTH domain or the C-terminal half of Glepsin comprising the methionine/glutamine/proline-rich (MQP) domain was driving aggregate formation in cells overexpressing the protein, the N-terminal (aa 1-158, construct GlepsinN-HA) and C-terminal parts (aa 218-406, construct GlepsinC-HA) of Glepsin were expressed individually in transgenic *Giardia* cells. GlepsinN-HA showed an even cytoplasmic distribution in fluorescence microscopy (Fig 1Bb). Conversely, GlepsinC-HA was only found as cytoplasmic aggregates by fluorescence microscopy. Aggregates were also detectable using bright field differential interference contrast (DIC) microscopy alone (Fig 1Bc). The subcellular localization of the aggregate(s) varied and they could occasionally be found in close proximity of ESVs (data not shown). Hence, strong inducible expression of Glepsin for 8 hours did not reveal any association with organelles, including ESVs, or structures of the endomembrane system in encysting trophozoites. Because strong ectopic expression of tagged Glepsin was found to produce aggregation, we used the endogenous Glepsin promoter to drive constitutive expression of an HA-tagged full length variant in order to avoid overexpression. Constitutive expression of a chromosomally integrated (Jimenez-Garcia *et al.*, 2008) construct encoding the full length tagged variant showed a distinct localization of the proteins at the ventral disk (VD) (Fig. 1Bd). This result could be reproduced by expression of an integrated copy under the control of a tubulin promoter (Elmendorf *et al.*, 2001) (Fig. 1 Be). When viewed from the dorsal or ventral side, the geometry the dome-shaped VD results in a signal distribution which gives the impression that most of the protein is localized at the rims of the VD (Figure 1 Bd, upper panel). However, imaging the VD from the side (90° angle) shows a much more even signal distribution (Figure 1 Bd, lower panel).

Polyglutamine stretches such as at the C-terminal MQP domain of GlEpsin are also found in the huntingtin protein which produces intracellular aggregates in people with Huntington's disease. Proteolytic cleavage of huntingtin causes a conformational change in the polyglutamine tract (Shao and Diamond, 2007) which may lead to formation of toxic oligomeric aggregates manifesting as macromolecular intracellular inclusions (Paulson *et al.*, 2000).

To determine whether the ENTH domain or the C-terminal MQP domain were responsible for targeting GlEpsin to the VD, the truncated versions GlepsinN-HA or GlepsinC-HA were expressed constitutively in transgenic cells under the control of the tubulin promoter. GlepsinC-HA localized at the VD in a pattern very similar to the full-length product albeit with a more punctate distribution (Fig. 1Bg), whereas the N-terminal part containing the ENTH domain was evenly distributed in the cytoplasm (Fig. 1Bf). Taken together, these data demonstrate that the GlEpsin C-terminal domain, which contains a high proportion of methionine, proline and glutamine residues, is necessary and sufficient for targeting Glepsin to the VD. Addition of the HA epitope at the N-terminus as opposed to the C-terminus of GlepsinFl did not change the construct's localization (data not shown).

Because the ENTH domain is the most conserved domain of the giardial epsin protein, we tested the ability of recombinant Glepsin variants produced in *E. coli* to bind to membrane lipids in a lipid strip binding assay. Incubation of the recombinant GlepsinN-HA fragment containing the ENTH domain with a lipid library spotted on strips (lipid strips) revealed phosphatidylinositol (3,4,5)-triphosphate (PtdIns (3,4,5)P3) as the main lipid bound by recombinant Glepsin (Fig. 1C), whilst binding to PtdIns (4,5)P2 was weak. Conversely, the recombinant GlepsinC-HA fragment did not

interact with any of the lipids tested. Interestingly, mammalian epsins were shown to interact with PtdIns (4,5)P2 in COS-7 cells whereas epsinR binds preferentially to PtdIns(4)P (Itoh *et al.*, 2001) in Golgi membranes.

Based on the localization data and the absence of detectable binding domains for clathrin, ubiquitin, or AP1 in Glepsin we propose the following testable working model for Glepsin function (Fig. 2): Glepsin binds to elements of the VD cytoskeleton and to lipids of the plasma membrane (PM), connecting both at a molecular level. This model is supported by the strong preference for targeting of the protein to the VD cytoskeleton which is determined by the MQP domain. A testable hypothesis is therefore that the comprised low complexity sequence interacts specifically with cytoskeleton components that are unique to the VD structure. Although the ENTH domain exhibits a strong preference for binding to PtdIns (3,4,5)P3, this feature does not appear to contribute to targeting of epitope tagged variants to the VD. However, as depicted in Fig. 2, this part of the protein may confer a physical link with the PM adjacent to the VD.

Interestingly, in trophozoites which were only briefly induced, conditionally expressed Glepsin-HA localized mostly to the cytoplasm and not to the VD structure. A possible explanation is that since these cells do not progress beyond G2, the newly made Glepsin-HA protein is prevented access to the space between the disk cytoskeleton and the PM. Hence, proper targeting of Glepsin to the VD cytoskeleton may require disassembly of the VD and rebuilding of daughter discs during cytokinesis. This fits with our observation that in proliferating transgenic trophozoites constitutively expressed Glepsin-HA localized specifically to the VD (Fig. 1Bv, vi).

In addition to binding to phosphoinositols, ENTH domain proteins as well as dynamin, have been shown to deform membranes and generate membrane tubules (Ford *et al.*, 2002). In contrast to dynamin, formation of membrane tubules by epsins is independent of oligomerization but is attributed to insertion of the alpha-0 helix of the ENTH domain into the outer leaflet of the lipid bilayer (Ford *et al.*, 2002). This induces membrane curvature and facilitates recruitment of accessory molecules onto the membrane. In analogy to this, our model for Glepsin function (Fig. 2) proposes that the protein provides a physical link between the concave VD and the PM. This interaction would be mediated by the Glepsin C-terminus with VD microtubules or microtubule-associated proteins (MTAP) on one side, and by insertion of the alpha-0 helix of the N-terminal ENTH domain into the inner layer of the PM on the other. This configuration may also help induce the necessary membrane curvature while effectively stitching the PM to the concave VD cytoskeleton and providing essential functionality to this highly specialized attachment organelle.

Sequestration of Glepsin at the outer face of the VD is consistent with the lack of conserved domains that allow canonical epsins to interact with membrane coat components. Hence, although AP complexes seem to play a role in giardial membrane transport (Rivero *et al.*, 2010), Glepsin does not appear to be involved in coat protein recruitment. This also fits with the absence of clathrin coated membrane buds or transport vesicles in *Giardia*. Consistent with this, an extensive co-immunoprecipitation study with giardial clathrin or AP components as bait proteins did not reveal Glepsin as an interaction partner (Zumthor & Hehl, unpublished).

Taken together, the Glepsin domain structure suggests that the protein is most likely a reduced epsinR variant with a conserved ENTH domain and methionine-rich elements that has lost most of the middle section including all motifs involved in recruitment of coat protein components. Given its distinctive localization at the VD in proliferating trophozoites and the demonstrated functionalities, we propose that reorganization and secondary loss in membrane trafficking pathways (Faso and Hehl, 2011) have led to a complete reassignment of its function. Glepsin is yet another striking example how protein domains with fundamental functions are conserved and reassigned if their original function(s) have become obsolete due to a lack of interaction partners and/or the elimination of entire pathways.

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Figure legends

Fig. 1. A) Domain structures of human epsin1 and epsinR homologues in comparison to Glepsin. The proteins share a highly conserved N-terminal ENTH domain. Epsin1 possesses several additional motifs, such as ubiquitin-interacting motifs, clathrin binding motifs as well as DPW motifs that bind to AP2, and NPFs motifs that bind to Eps15. EpsinR proteins have no ubiquitin-interacting motifs (UIM) or asparagine-proline-phenylalanine (NPF) repeats. However, they possess an additional AP/GGA binding motif as well as a methionine rich C-terminal domain with unknown function. The giardial Glepsin harbors no UIM or NPF domains and is structurally most similar to epsinRs including a low complexity domain enriched in methionine, proline and glutamine residues (MQP). B) Subcellular localizations of Glepsin variants. Representative localization of C-terminally HA-tagged variants after inducible (8 hours) (pCWP1) or constitutive (pEndo, pTub) expression (wide field microscopy). FITC-conjugated rat anti-HA antibody (green) was used to detect HA-tagged Glepsin variants. Nuclear DNA was labeled with DAPI. The table on the right side schematically depicts the localization pattern of Glepsin (abbreviations: pCWP1: inducible CWP1 promoter; pendo: endogenous promoter; DPW, NPF and GGA represent amino acid residues; Scale bar: 10 μm). C) Lipid overlay assay and detection of recombinant, HA-tagged Glepsin fragments. GlepsinN-HA binds to phosphatidylinositol (3,4,5)-trisphosphate. GlepsinN-HA or GlepsinC-HA were expressed in *E. coli* and incubated with membrane lipid strips (Echelon Biosciences, Salt Lake City, Utah, United States). Recombinant protein was detected by luminescence assay using a primary rat-derived anti-HA antibody and HRP-conjugated secondary goat anti-rat antibody.

Fig. 2. Model showing Glepsin in its function as a linker between the microtubule array of the ventral disk cytoskeleton and the plasma membrane underlying the disk in *Giardia lamblia,* possibly via an unknown microtubule adaptor protein (MTAP). Nucleus depicted as gray circle.